IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE APPLICATION OF	: Mitsuko Ishihara et al.
SERIAL NUMBER	: 09/892,485
FOR	: MEHTOD FOR DETECTING ENDOCRINE DISRUPTING ACTION OF A TEST SUBSTANCE
FILED	: June 28, 2001
GROUP ART UNIT	: 1655
EXAMINER	: Arun K. Chakrabarti

DECLARATION UNDER 37 C.F.R. 1.132

Assistant Commissioner for patents
Washington, D.C. 20231

Sir:

I, Mitsuko Ishihara, a national of Japan, hereby declare and state as follows.

I received a Bachelor of Science in Biology from the Tokyo Science University in March 1989.

I joined KABUSHIKI KAISHA TOSHIBA in April 1989, and have been employed, since then at the company's Corporate Research & Development Center, where I have been engaged in research and development of assessment system for environmental pollutants.

I am a co-inventor of the present invention described and claimed in the above-identified application.

I have conducted the following experiments:

1. Culture of Neuro2A

Neuro2A (purchased from ATCC) was cultured in a culture flask by means of adhesion culture using a culture medium obtained by mixing, at 10%, a fetal bovine serum FCS (a product of Dainippon Pharmaceutical Co., Ltd.) from which T3 was removed in advance, to a DF culture medium (a mixture of Dulbecco's MEM medium and ham F-12 medium at a ratio of 1 : 1). The culture was carried out under conditions of 37℃ and 5%CO_2. The removal of T3 from the fetal bovine serum was carried out by a batch process with use of an anionic exchange resin (AG1-X8, a product of Bio-Rad). (See the specification of the present invention,

Example 1-1.)

2. Exposure of 2,3,7,8-TCDD

The cells obtained above were incubated for two days under conditions of adding and not adding 30 nM T3 to the cells. Then, thus cultured cells were divided into the following condition groups, and each group was further incubated for 24 hours with addition of TCDD (2,3,7,8-tetrachloro-*p*-dibenzo-dioxin) thereto. (See the specification of the present invention, Example 1-2.)

(A) cells + 30nMT3 + 1nMTCDD

(B) cells + 30nMT3

(C) cells + 1nMTCDD

(D) cells only

3. Extraction of RNA

From each cell incubated under the above conditions (A) to (D), mRNA was extracted with use of an RNA extraction kit (a product of Amersham Bio Science). More specifically, to the cells ($5x10^6$), 18ml of homogenate buffer (containing guandineisocyanate, SDS, EDTA) was added to homogenize them, and then the homogenate was centrifuged at 5000g for 20 minutes to remove the sedimentation. The supernatant was allowed to pass a G syringe 10 times, and overlaid on a cesium solution (containing CsTFA and EDTA) of the same amount. The resultant was then subjected to ultracentrifugation at 30,000 g for 20 hours. The obtained supernatant was taken out carefully, and the residual was dried. Then, the sediment was washed with 70% ethanol and allowed to dry. Lastly, the sediment was suspended in 50 μ l of distilled water. (See the specification of the present invention, Example 3-1.)

4. Detection with use of PCR (FDD)

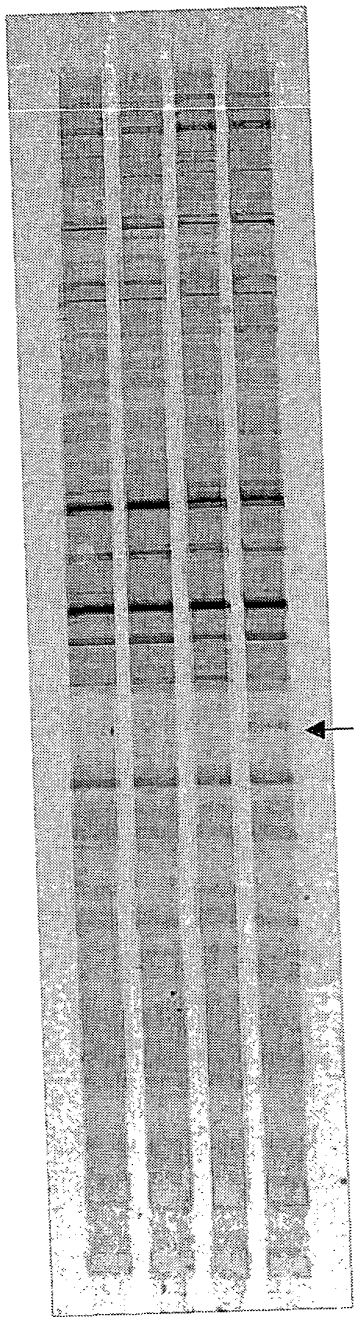
The RNAs obtained as above and expressed under various conditions were compared by FDD based on PCR. The FDD (fluorescence differential display) was carried out with use of Takara FDD kit Fluorescent version (Takara). That is, to RNA (1 μ g), RT-buffer,

dNTP Mix (10uM), RNase inhibitor (10U), Primer (25 pmol) and AMV RTaseXL (5U), water was added to make a final amount of 20 μl. The primer was (TTTTTTTTTTTTAA) and its end was labeled with a fluorescent dye, rhodamine. This solution was treated at 55℃ for 30 minutes and then at 99℃ for 5 minutes to synthesize c DNA. To the resultant solution, PCR buffer, dNTP (10 μM), MgCl2 (1.25mM), LA-Taq (2.5U) and a primer were added to make a final amount of 100ul. The primer used here was different from the fluorescent primer mentioned above, and was an upstream primer (GATCATAGCC). The solution was then dispensed into portions of 20 μl, which were set on a thermal cycler, and subjected to cycle amplifications at cycles of 94℃ for 30 seconds, 45℃ for 1 minute, 72℃ for one minute. After, the amplifications, the PCR solutions (A to D) were loaded on lanes adjacent to each other, and separated with 4%PAGE. The gel obtained after the electrophoresis was scanned with a fluorescent image scanner (Typhoon: product of Amersham Bio Science) to detect the band for each solution. (See the specification of the present invention, Example 3-2.)

Results of the Experiments

The results of the electrophoresis obtained by the above-described experiments are shown in the photograph attached here. As shown in the photo, the expression of a gene that is present only in lane (A) was observed, and this fact indicates that dioxin has an endocrine disrupting action.

(D) (C) (B) (A)



I, the undersigned, declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

Date July 24, 2003

Mitsuko Ishihara

Mitsuko Ishihara